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REACTIONS OF HYPOCHLORITE AND ORGANIC N-CHLORAMINES IN STOMACH FLUID

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16. ABSTRACT

Over the past ten years it has been recognized that chlorine, used to disinfect drinking water, reacts with trace organic compounds dissolved in natural waters to produce by-products which may have adverse health effects in man. However, little attention has been given to the possible reactions of hypochlorous acid which may take place in the organic-rich medium of the stomach on ingestion of chlorinated drinking water. The average person's daily diet includes a minimum of 30 to 45 grams of protein. Through the action of digestive enzymes in the stomach proteins are broken down into peptones, large polypeptides, and about 15% amino acids. Therefore, a variety of amino nitrogen compounds are avilable substrates for reaction with aqueous hypochlorous acid in the stomach. It was the objective of the study described here to examine the possibility that organic N-chloramines could be formed on ingestion of hypochlorous acid and to determine whether these compounds could be absorbed into the bloodtsream for circulation to other parts of the body.

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INTRODUCTION

Over the past ten years it has been recognized that chlorine, used to disinfect drinking water, reacts with trace organic compounds dissolved in natural waters to produce by-products which may have adverse health effects in man. However, little attention has been given to the possible reactions of hypochlorous acid which may take place in the organic-rich medium of the stomach on ingestion of chlorinated drinking water. The average person's daily diet includes a minimum of 30 to 45 grams of protein. Through the action of digestive enzymes in the stomach proteins are broken down into peptones, large polypeptides, and about 15% amino acids.[1] Therefore, a variety of amino nitrogen compounds are available substrates for reaction with aqueous hypochlorous acid in the stomach. It was the objective of the study described here to examine the possibility that organic N-chloramines could be formed on ingestion of hypochlorous acid and to determine whether these compounds could be absorbed into the bloodstream for circulation to other parts of the body. Two chloramines were selected to probe the reactions of hypochlorite and the stabilities of chloramines in the stomach, N-chloroglycine and N-chloropiperidine. The first is a relatively stable chloramino acid[2] which is likely to be present in the stomach because of ingestion or as a product of proteolytic activity. The second is a model of more stable chloramines. The parent amine, piperidine, has been identified in drinking water[3] and its N-chloro derivative, N-chloropiperidine, has been found to be mutagenic.[4]

Recently, a method for the derivatization and analysis

of organic N-chloramines in dilute aqueous solution was described.[5] In the method solutions containing N-chloramines were reacted with 5-dimethylaminonaphthalene-1-sulfinic acid ($DANSO_2H$) to produce highly fluorescent sulfonamide derivatives (dansyl derivatives) which could be analyzed by high performance liquid chromatography (HPLC). In the present study this derivatization method is used to detect the formation of N-chloroglycine in stomach fluid, but results are corroborated by chromatography of an underivatized chloramine (N-chloropiperidine) and its radiolabelled counterpart.

EXPERIMENTAL

General

The method used for the derivatization of organic N-chloramines and the liquid chromatographic equipment used in these studies has been described elsewhere.[5] [^{14}C(U)]-glycine was purchased from New England Nuclear and Amersham Corporation. [G-^{3}H]piperidine hydrochloride was purchased from Amersham Corporation. Radiochemicals were greater than 98% pure. Solutions of sodium hypochlorite were prepared and standardized as described in EPA Method 510.1. Total Kjeldahl nitrogen determinations were performed according to EPA Method 351.3 using titration with standard sulfuric acid. Protein concentrations were determined by the biuret method.[6] A standard curve was constructed using bovine serum albumin (BSA) as the standard.

Synthesis of Tritiated N-Chloropiperidine

^{3}H-piperidine (200 µL of a 1 µCi/µL solution) was added dropwise with stirring to 2.2 mL of an ice-chilled aqueous solution of sodium hypochlorite (2000 mg/L as Cl_2). Unlabelled piperidine (200 µL of a 0.2 M solution) was added with stirring to produce the solution used in animal studies or in exchange experiments.

Animals

Adult Sprague-Dawley rats weighing between 250 and 400 grams were starved for 48 hours before each experiment. Prior to administration of a solution by gavage, the animals were anesthetized with ether. All solutions were administered by gavage using a syringe fitted with a 3 in. curved intubation needle.

Dosing and Recovery of Stomach Fluid

The contents of the stomach were recovered within 3 to 4 minutes after administration of a test solution by removal of the whole stomach. The fluid was chilled immediately on ice. Centrifugation was occasionally necessary to remove particulate matter before analysis was carried out.

In order to characterize the concentration of nitrogen compounds in the stomachs of test animals, 4 mL of deionized water were routinely administered.

To determine whether organic N-chloramines could be formed in stomach fluid, solutions of sodium hypochlorite (4 mL) with concentrations of either 200 mg/L or 1000 mg/L were administered

by gavage. Within 30 min. of recovery the stomach fluid was derivatized with 5-dimethylaminonaphthalene-1-sulfinic acid ($DANSO_2H$).[5]

When the formation of either N-chloropiperidine or N-chloroglycine in stomach fluid was examined, dosing of animals was carried out in two stages. Initially 0.5 or 1.0 mL of an amine solution (0.2 M piperidine or 0.5 M glycine) in water was administered. This was followed within 2-3 min. by a sufficient volume of standardized hypochlorite (200 mg/L or 1000 mg/L) to give a total administered volume of 4.0 mL. Stomach fluid containing N-chloroglycine was passed through a Waters Assoc. C_{18} SEP-PAK cartridge and the chloramine recovered by washing the cartridge with 2.0 mL of a solution of 20% acetonitrile/80% water (1% acetic acid). The wash was immediately derivatized with $DANSO_2H$. The procedure for handling stomach fluid containing N-chloropiperidine was similar except the chloramine was recovered using a 1.0 mL wash of acetonitrile. The chloramine was analyzed without derivatization.

When the appearance of N-chloropiperidine in plasma was examined, 1.2 mL of a 0.017 M N-chloropiperidine solution containing tritiated N-chloropiperidine (100 microcuries) was administered by gavage. Animals were sacrificed at 30, 60, and 120 minutes to obtain blood. The blood was centrifuged to remove red blood cells and proteins were removed from the plasma by alcohol precipitation at -80 ^{o}C. Samples were then concentrated and analyzed directly by liquid chromatography.

Chlorine Demand of Stomach Fluid

After recovery of stomach fluid from animals administered 4 mL of deionized water, 1.5 mL of the fluid was diluted to 100 mL with deionized water, and 20 mL aliquots were chlorinated to 5, 10, 15, 20, and 30 mg/L Cl_2. Samples were incubated in the dark at room temperature for one hour before being analyzed for residual chlorine by the DPD titrimetric method. A breakpoint curve was constructed and the chlorine demand in mg/L as N determined by dividing the amount of chlorine required to reach breakpoint by 10, since this is the Cl_2 : N weight ratio at which glycine reaches a breakpoint.

Chromatographic Conditions

Chromatographic conditions for the analysis of dansylglycine have been described.[5] The gradient program used to analyze samples containing radiolabelled N-chloropiperidine included isocratic elution at 10% acetonitrile/90% H_2O (1% acetic acid) for the first five minutes. During this time the solvent flow was programmed from 0.5 to 2.0 mL/min. After five minutes the solvent was programmed by a linear gradient over the next ten minutes up to 30% acetonitrile (constant flow). For the remaining five minutes of the chromatography the solvent was programmed from 30% to 50% acetonitrile (constant flow).

RESULTS AND DISCUSSION

Amino Nitrogen Compounds in Stomach Fluid

Typical protein and total Kjeldahl nitrogen (TKN) concentrations of the stomach fluid in laboratory rats have been measured and results are listed in Table I. It was necessary to acquire these data so that experiments involving various dosage levels of hypochlorite could be designed with a clear understanding of the effect these levels would have on the stability of organic amino nitrogen compounds in the stomach. Since N,N-dichloro derivatives of primary amines are considerably less stable than their mono-chloro counterparts, administration of too high a concentration of hypochlorite would cause decomposition of the N-chloro compounds that later experiments were designed to detect.

The protein concentrations as determined by the biuret method are recorded in Table I in mg/L as BSA (bovine serum albumin). If the percentage nitrogen in the stomach proteins is assumed to be identical to the percent nitrogen in the BSA (15.7%) used to construct the standard curve, then the protein contribution to the TKN value can be estimated. The results of this calculation suggest that 70 - 99% of the TKN nitrogen is proteinaceous. In addition, protein nitrogen concentrations in the stomach fluid of rats (as calculated from the biuret results) range from 100 mg/L to 400 mg/L. These values have not been normalized for the weight of the animal for which they were determined. However, variations between animals are not believed to be due to weight differences, but rather to the

amount of food remaining in the stomach at the time the fluid was acquired as well as metabolic differences between animals.

The values measured for the chlorine demand of rat stomach fluid are listed in the last column of Table I as mg/L as N. They were obtained from breakpoint curves constructed from the chlorine residuals measured one hour after chlorinating diluted samples of stomach fluid. The curves showed a definite maximum followed by a relatively large "irreducible minimum," beyond which free residual chlorine was measured. The shape of the curve looked much like that obtained with model solutions containing proteins and amino acids. Chlorine demand in stomach fluid can be exerted by many different species: sulfhydryl residues, amino nitrogen end groups and side-chains on proteins, reducing sugars, trace metals, and other reducing species. If all of the demand is assumed to be exerted by amino nitrogen moieties, a maximum value for the contribution of amino nitrogen to the TKN measurement can be calculated. Although these values vary considerably, they are generally less than one-half of the TKN values. This also suggests that a large portion of the nitrogen is found within proteins and peptides. Taking an average of the values for the chlorine demand (in mg/L as N) gives 74 mg/L. Since a 1 : 1 ratio of chlorine : nitrogen would be achieved at a chlorine concentration of about 370 mg/L, chlorine concentrations higher than this would result in decomposition of N-chloro derivatives of simple primary amines and amino acids.

In Vivo Formation of Stomach N-Chloramines

Laboratory rats were administered 4 mL sodium hypochlorite (1000 mg/L), the stomach fluid was recovered shortly thereafter and derivatized with $DANSO_2H$. HPLC analysis of the resulting solution yielded the chromatogram shown in Figure 1. There are several major peaks in the chromatogram. The peak with a retention time of about 3 min. is due to unreacted $DANSO_2H$ and the peak with a retention time of 21 min. is an artifact due to oxidative degradation of the derivatizing reagent at high concentrations of hypochlorite or chloramines.[5] The remaining peaks in the chromatogram are believed to be derivatives of chloramines formed by derivatization of N-chloramines produced in the stomach when chlorinated water was administered. The compounds which appear in the chromatogram probably have relatively low molecular weights, since large proteins and polypeptides are retained on the octadecylsilica cartridge used in the clean-up step. Consequently, there are likely to be other N-chloramines formed on the side-chains of proteins which are not detected here. There is conspicuous absence of any significant amount of derivative of NH_2Cl (retention time = 10.5 min). This suggests that ammonia does not contribute significantly to the breakpoint curve of stomach fluid which was discussed above.

Because of the complex nature of stomach fluid, speculation about the identities of the compounds in the chromatogram by correlation of their retention times with those of known derivatives is not satisfactory. Therefore, the identity of an N-chloro derivative of a known amine was examined.

In Vivo Formation of N-Chloroglycine

Administration of a solution of glycine (0.5 M) followed by a solution of hypochlorite (either 200 mg/L or 1000 mg/L) resulted in formation of N-chloroglycine. The N-chloroglycine was detected by derivatization with $DANSO_2H$ and HPLC analysis (Figure 2, dansyl glycine retention time = 14 min.). When the 1000 mg/L solution of hypochlorite was used, the amount of dansyl glycine formed was found to be proportionally higher than the amount of dansyl glycine formed when the 200 mg/L solution was used.

Additional confirmation of N-chloroglycine formation was obtained using ^{14}C-glycine. Rats were dosed with 10 to 12 microcuries of the radiolabelled glycine in a total volume of 1 mL and then with hypochlorite. Approximately 40 to 50% of the radiolabel was routinely recovered from the stomach. The derivatized extract was separated by HPLC and fractions were collected and analyzed by liquid scintillation counting. When 1000 mg/L hypochlorite was used, approximately 4.5% of the radiolabel eluted with the retention time of dansylglycine (Figure 3). When 200 mg/L hypochlorite was used, only 0.44% of the radiolabel which was recovered was present in the fraction which eluted with the retention time of dansylglycine. The low recovery is probably due to a combination of factors: absorption of N-chloroglycine or glycine into stomach tissues, and decomposition of the N-chloroglycine in vivo and during work-up.

In Vivo Chlorination of Piperidine

The derivatization technique which is used to detect the formation of N-chloroglycine in stomach fluid as described above is subject to the criticism that the derivatization is carried out at high pH (pH 9-10) and may not reflect what happens in the stomach at lower pH. Since N-chloropiperidine can be separated from its unchlorinated amine by liquid chromatography, the analysis of this chloramine is possible without derivatization. Elution of N-chloropiperidine from the chromatography column can be detected by its UV absorbance at 254 nm. For low concentrations of N-chloropiperidine, radiolabelled N-chloropiperidine can be used.

In general, the in vivo chlorination of piperidine was carried out in a manner similar to the chlorination of glycine. An aqueous solution of piperidine (0.5 mL of a 0.2 M solution) containing 12 microcuries of ^{3}H-piperidine was administered to the test animals shortly before dosing with hypochlorite. However, when the stomach fluid was isolated, only 30% of the radiolabel could be recovered. The material which was not recovered was believed to be absorbed into tissue. Approximately 30-40% of the recovered label was lost in the clean-up of the fluid with an octadecylsilica cartridge (SEP-PAK). The radiochromatogram of the labelled material which was recovered when a 1000 mg/L solution was used (Figure 4) showed that 8% was N-chloropiperidine. When 220 mg/L hypochlorite was used, 3% of the recovered material was N-chloropiperidine. Using the average chlorine demand of the stomach fluid estimated above (74 mg/L)

and adding it to the demand of the piperidine administered, an expected yield of N-chloropiperidine can be calculated. Such a calculation suggests that 19% of the piperidine should be chlorinated when 3 mL of 1000 mg/L hypochlorite is administered and 4% of the piperidine should be chlorinated when 3 mL of 220 mg/L hypochlorite is administered. The yields actually recovered (8% and 3%) may be low due to some decomposition of the chloramine in the stomach but may only appear low due to inadequate recovery of the chloramine during work-up. In any case, the actual recovered yields suggest not only that the formation of N-chloropiperidine is an important reaction of the hypochlorite in the stomach in this experiment, but also that the formation of other N-chloramino nitrogen compounds in the stomach probably takes place as well.

Absorption of N-Chloropiperidine into Blood Plasma

Since organic N-chloramines were shown to be formed in the stomach, an experiment was designed to determine whether they could be absorbed into the bloodstream and possibly distributed to other parts of the body. Animals were administered 100 microcuries of ^{3}H-N-chloropiperidine. Of the 100 microcuries administered only a small amount (adjusted for the entire volume of the plasma) appeared in the plasma (1 to 3 microcuries) at these intervals. Of the radiolabelled material found in the plasma the major portion was unchlorinated ^{3}H-piperidine. However, 5.5%, 16%, and 1% of the labelled material recovered at 30, 60, and 120

min., respectively, was due to the presence of tritiated N-chloropiperidine.

Conclusion

This study has demonstrated 1) that organic N-chloramines can be formed in stomach fluid when water containing high concentrations of hypochlorite is ingested and 2) that these chloramines can be absorbed into the bloodstream. However, several facts must be considered before the implications of these results on the health effects of drinking chlorinated water can be made. For instance, the high dosage levels used could be overwhelming any reducing mechanisms which might otherwise function to deactivate chlorine or chloramines at lower concentrations in the stomach. The low yields of chloramines recovered in blood plasma may be evidence that such mechanisms are surprisingly effective even at high chlorine dosages. On the other hand, absorption of the chloramine into stomach tissue or exchange of the active chlorine between the test chloramine and unchlorinated amines in the stomach may also account for the low concentration of the model chloramine in the plasma. The mechanisms by which active chlorine compounds are decomposed in the stomach will require further study.

Finally, a single administration of chlorine or a test chloramine is not an adequate model of the daily ingestion of chlorinated water. Work is currently underway to evaluate the effect of multiple administration of active chlorine compounds.

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Table I. Protein and Nitrogen Concentrations in Rat Stomach Fluid

ANIMAL NO.	PROTEIN[a] (mg/L as BSA)	PROTEIN-N[b] (mg/L as N)	TKN (mg/L as N)	CHLORINE DEMAND (mg/L as N)
1	1340	201	208	125
2[c]	2745	412	415	
3[c]	1780	267	357	
4	2112	317	307.5	
5	800	120	171.7	42
6	740	116		45
7	2430	382		83
8	2732	429		60
9	2940	462		41
10	2318	364		73
11	1828	287		73

[a]By biuret method; [b]0.157 x protein as BSA; [c]Composite sample

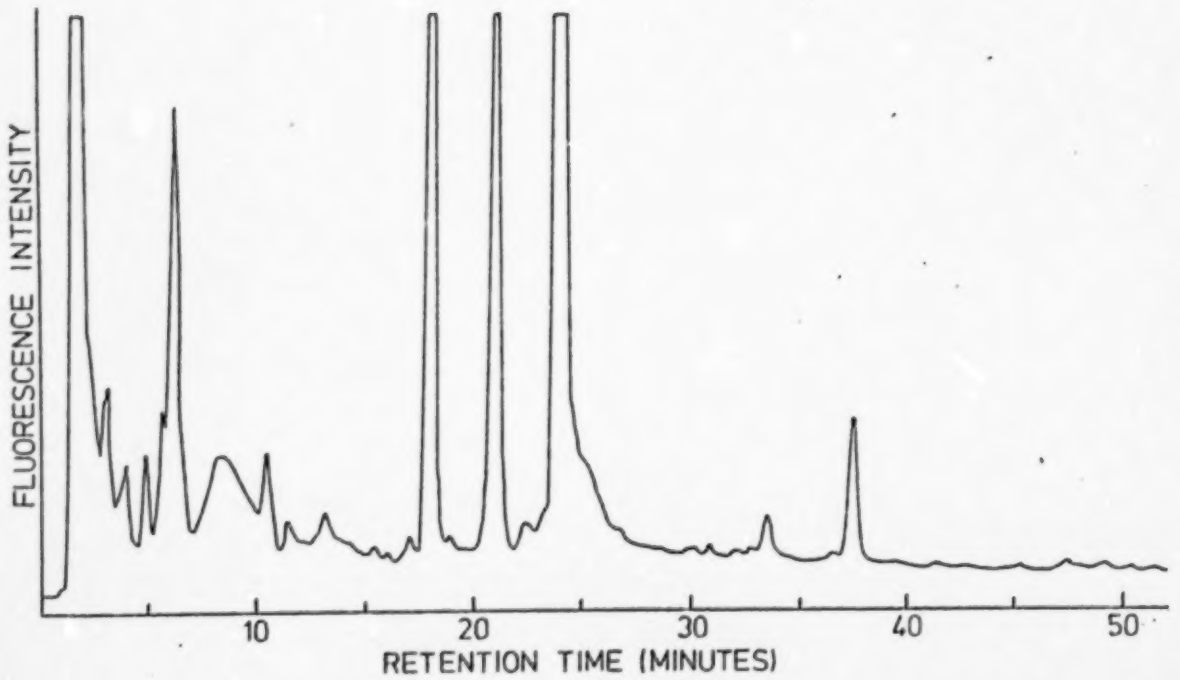

Figure 1. Chromatogram of stomach fluid from a rat which had been given 4 mL of a solution of hypochlorite (1000 mg/L) five minutes before removal of the stomach contents. The fluid was derivatized with $DANSO_2H$ before chromatography. Injection size = 20 µL. The 21 min. peak is an artifact.

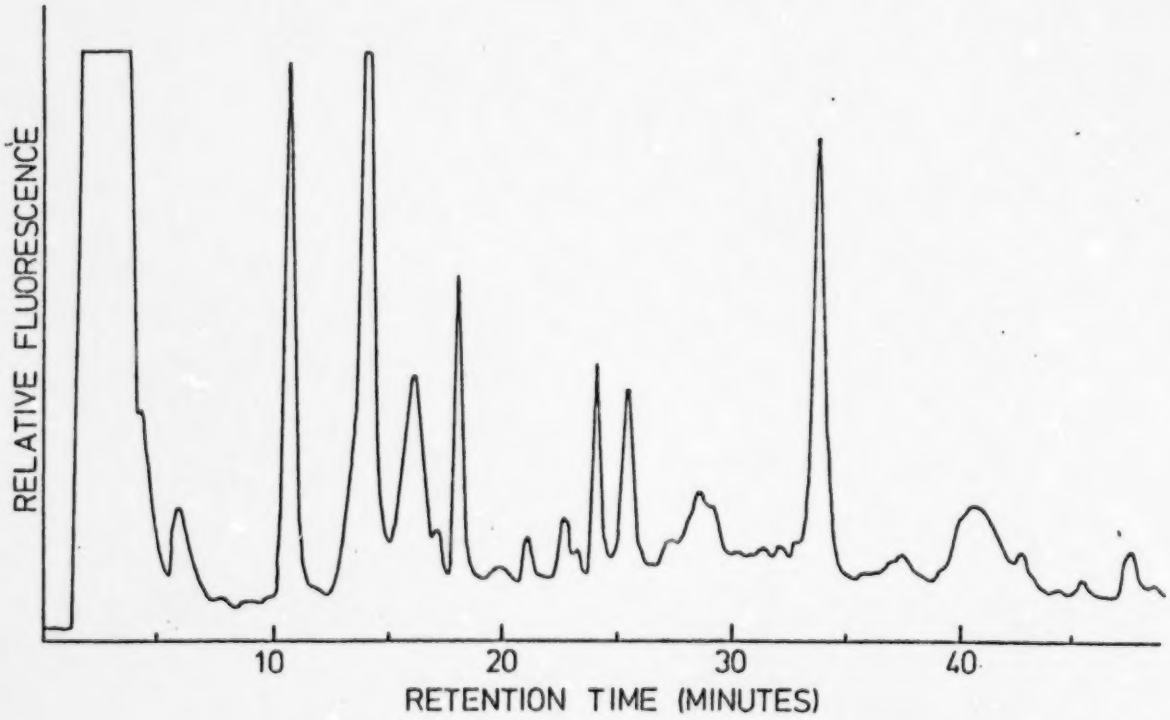

Figure 2. Chromatogram of stomach fluid from a rat which had been administered 0.5 mL of a 0.5 M solution of glycine followed by 3.5 mL of a solution of hypochlorite (1000 mg/L). The fluid was derivatized with $DANSO_2H$ before chromatography. Injection size = 37 µL. Dansylglycine has a retention time of 14 min.

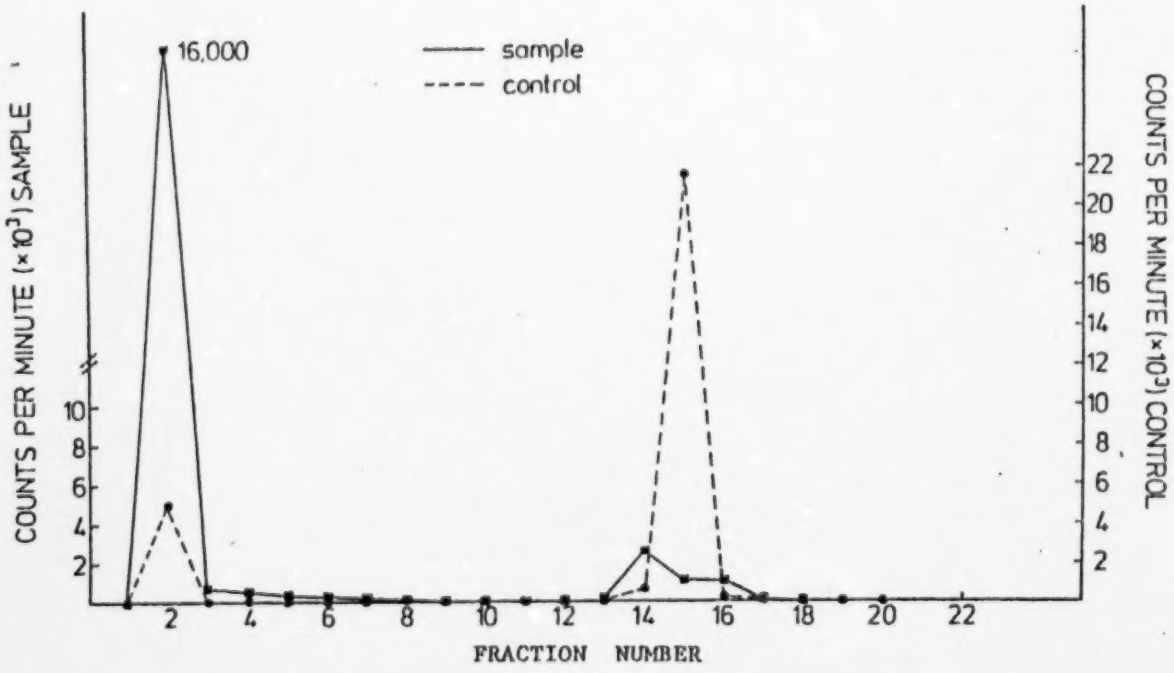

Figure 3. Radiochromatogram of stomach fluid from a rat which had been given 1 mL H_2O containing 12 µCi ^{14}C-glycine by gavage followed by 3 mL of a solution of hypochlorite (1000 mg/L). The fluid was derivatized with $DANSO_2H$ before chromatography. Fraction size = 1 mL. The radiochromatogram of ^{14}C-glycine derivatized with dansyl chloride (•------•) was used to check the purity of the labelled glycine. Dansylglycine elutes in fractions 14 to 17.